

REMARKS

Applicants have carefully studied the Final Office Action dated June 23, 2009, which issued in connection with the above-identified application. The present response is intended to be fully responsive to all points raised by the Examiner and is believed to place the claims in condition for allowance. Favorable reconsideration and allowance of the present claims are respectfully requested.

December 8, 2009 Telephonic Interview with Examiners Wang and Saoud

Applicants gratefully acknowledge the courtesy shown by Examiner Chang-Yu Wang and the Examiner's Supervisor, Christine Saoud, during a telephonic interview with applicants' representatives, Irina Vainberg, Sybil Lombillo and William Chiang, and inventor, Dr. Dan Felsenfeld, on December 8, 2009. During the interview, proposed amendments to the claims were discussed and applicants' representatives and Dr. Felsenfeld provided arguments as to the lack of anticipation and non-obviousness of the present claims over Tuvia et al. (Proc. Natl. Acad. Sci. USA, 1997, 94:12957-12962) and Garver et al. (J. Cell. Biol., 1997, 137:703-714).

During the interview, Examiners indicated that the proposed amendments should overcome the outstanding rejections under 35 U.S.C. § 112, first paragraph, and suggested direction for additional amendments to claim 6 to overcome anticipation and obviousness rejections over the Tuvia and Garver references. Applicants believe that the amendments introduced herein are in line with the Examiners' suggestions provided during the interview.

Pending Claims

Claims 1-4 and 6-25 were pending and at issue in this application. Claims 1-4, 8-17 and 25 have been withdrawn from consideration as drawn to a non-elected invention. Claims 20 and 24 have been rejected for lack of enablement. Claims 6, 7, 18-19, and 21-23 have been rejected as being anticipated and/or obvious over prior art.

To expedite prosecution, withdrawn claims 2-4, 8-17 and 25 have been canceled without prejudice or disclaimer. Applicants preserve the right to prosecute the subject matter of these claims in a continuing/divisional application.

Claims 6, 7, 19, 20, 23, and 24 have been amended to correct typographical errors to replace the article "an" with "the".

Claims 20 and 24 have been also re-written in independent format.

Following the Examiners' suggestion during the interview of December 8, 2009, claim 6 has been further amended to clarify that the claimed peptide "does not contain transmembrane and extracellular portions of the L1-CAM family member protein" and "comprises the amino acid sequence of QFNEDGSFIGQF (SEQ ID NO: 2)". Support for these amendments can be found, for example, in paragraphs [0053-0055], [0057], Example 8 (paragraphs [0166-0172]), and Figures 6 and 12 of the application as published (US Publication No. 20060142189). While the words "does not contain transmembrane and extracellular portions of the L1-CAM family member protein" literally do not appear in the present specification, this negative limitation satisfies the requirements of 35 U.S.C. § 112, first and second paragraphs, under MPEP § 2173.05(i). Specifically, Figure 12 of the present application demonstrates that while the L1-CAM family members contain extracellular, transmembrane and cytoplasmic portions, ankyrin binding involves only the cytoplasmic portion of these molecules and does not include the extracellular and transmembrane portions. Paragraph [0053] of the present application further specifies that the peptides of the invention are derived from the "region of the L1-CAM cytoplasmic tail that has been shown to be required for ankyrin binding" (emphasis added) and provides a reference to Zhang et al., J. Biol. Chem., 1998, 273:30785-30794. Zhang et al. (attached as Exhibit A), which is incorporated by reference in the present application in its entirety, in turn, discloses that the ankyrin binding domain corresponds to a highly conserved sequence from Ser⁵⁶ to Tyr⁸¹ in the cytoplasmic domain of neurofascin (L1-CAM family member) and does not include extracellular and transmembrane

portions (see, e.g., p. 30785 right col., second par. and Fig. 5 at p. 30789)¹. Thus, the present application provides support for the genus of peptides which are derived from the ankyrin binding domain of an L1-CAM family member protein and do not contain transmembrane and extracellular portions of the L1-CAM family member protein.

Claim 19 has been further amended to specify that the claimed peptide *consists essentially of*² “(i) the ankyrin binding domain of an L1-CAM family member protein or a fragment of such ankyrin binding domain, wherein the carboxy-terminal tyrosine is substituted with phenylalanine and which comprises the amino acid sequence of QFNEDGSFIGQF (SEQ ID NO: 2) and (ii) optionally, a targeting sequence which allows translocation of the peptide across the plasma membrane and into the cytoplasm of cells.” Similarly to claim 6, support for these amendments can be found, for example, in paragraphs [0053-0055], [0057], Example 8 (paragraphs [0166-0172]), and Figures 6 and 12 of the application as published. Specifically, paragraph [0053] of the present specification discloses that the peptides of the invention are “derived from the ankyrin binding domain of the L1 family members.” Zhang et al. (attached as Exhibit A and incorporated by reference in the present application in its entirety) discloses that the ankyrin binding domain corresponds to a highly conserved sequence from Ser⁵⁶ to Tyr⁸¹ in the cytoplasmic domain of neurofascin (L1-CAM family member) (see, e.g., p. 30785 right col., second par. and Fig. 5 at p. 30789). Thus, a shorter peptide QFNEDGSFIGQF (SEQ ID NO: 2) disclosed in the present application constitutes a fragment of the ankyrin binding domain, wherein the carboxy-terminal tyrosine is substituted with phenylalanine.

No new matter has been added as a result of these amendments.

¹ Same boundaries for the ankyrin binding domain are provided in the Garver reference cited by the Examiner (Garver et al., J. Cell. Biol., 1997, 137:703-714; see Fig. 3 and corresponding disclosure at p. 708, left col., top).

² Applicants respectfully note that, as specified in MPEP § 2111.03 and cases cited therein, the transitional phrase “consisting essentially of” is different from the transitional phrase “comprising” in that it limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s) of the claimed invention.

Rejoinder of Non-elected Claim 1

Withdrawn claim 1 recites a method for promoting outgrowth of a mammalian neuron comprising contacting said neuron with the peptide of claim 6. Thus, claim 1 depends from claim 6.

Upon the allowance of the linking claim 6, applicants respectfully request rejoinder of withdrawn claim 1.

Applicants point out the Examiner's statement in the Office Action dated September 17, 2008, that, where applicants elect claims directed to the product, and the product claims are subsequently found allowable, withdrawn process claims that depend from or otherwise require all the limitations of the allowable product claims will be considered for rejoinder and fully examined (see page 3 of the Office Action dated September 17, 2008).

Claim Rejections - 35 USC § 112

Claims 20 and 24 have been rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. The Examiner contends that, while an isolated peptide comprising or consisting of the amino acid sequence of SEQ ID NO:2 or a fusion protein consisting of the amino acid sequence of SEQ ID NO:2 fused to the amino acid sequence of SEQ ID NO:6 are disclosed and enabled in the present application, claims 20 and 24 as currently pending encompass any fragment derived from SEQ ID NO: 2 or SEQ ID NO: 3 and are therefore not enabled. The Examiner similarly interprets claims 6, 7, 19, and 23 as encompassing fragments of SEQ ID NO: 2 or SEQ ID NO: 6.

Without admitting the correctness of the Examiner's rejections and solely to expedite prosecution, claims 6, 7, 19, 20, 23, and 24 have been amended to replace the article "an" with "the". Claim 6 has been further amended to clarify that the claimed peptide comprises the amino acid sequence of QFNEDGSFIGQF (SEQ ID NO: 2).

In light of the above amendments, withdrawal of the rejections under 35 U.S.C. § 112, first paragraph, is respectfully requested.

Claim Rejections - 35 USC § 102

Claims 6, 18, 19, 21, and 22 have been rejected under 35 U.S.C. § 102(b) as anticipated by either Tuvia et al. (Proc. Natl. Acad. Sci. USA, 1997, 94:12957-12962) or Garver et al. (J. Cell. Biol., 1997, 137:703-714) as evidenced by Davis et al. (J. Cell. Biol., 1996, 135:1355-1367). The Examiner argues that claims as amended still encompass the full-length L1-CAM family member proteins disclosed in the Tuvia and Garver references despite the claim wording that the peptide is "derived from the ankyrin binding domain of an L1-CAM family member protein." The Examiner also states that the Tuvia and Garver references anticipate the claims, because they disclose neurofascin and L1 proteins, which are able to promote neurite outgrowth.

In response, applicants respectfully note that claims 6 and 19 recite an isolated peptide which comprises the amino acid sequence of QFNEDGSFIGQF (SEQ ID NO: 2). The *wild-type* full-length L1-CAM family member proteins disclosed in the Tuvia and Garver references do not comprise the sequence QFNEDGSFIGQF as required by the claims.

Applicants further respectfully note that claims 6 and 19 have been amended to explicitly exclude the full-length FIGQY->F mutant neurofascin disclosed in the Tuvia and Garver references or other full-length L1-CAM family member proteins.

Claims 18, 21 and 22 depend from claim 6 or 19 and thus also exclude the full-length FIGQY->F mutant neurofascin disclosed in the Tuvia and Garver references or other full-length L1-CAM family member proteins.

It follows, that none of the references cited by the Examiner discloses a peptide which has all characteristics required by the present claims.

In light of the above arguments, withdrawal of the anticipation rejection is respectfully requested.

Claim Rejections - 35 USC § 103

Claims 6, 7, 18-19, and 21-23 have been rejected under 35 U.S.C. § 103(a) as being obvious over Tuvia or Garver as evidenced by Davis in view of U.S. Patent No. 6,025,140 (Langel et al.) for the same reasons as provided for the anticipation rejection, above.

As explained in connection with the anticipation rejection, above, none of the cited references (even if taken together, and including secondary references) disclose or suggest an isolated peptide which has all characteristics required by the present claims. Thus, the *wild-type* full-length L1-CAM family member proteins disclosed in the Tuvia and Garver references do not comprise the sequence QFNEDGSFIGQF as required by the claims. Also, claims 6 and 19 have been amended to explicitly exclude the full-length FIGQY->F mutant neurofascin disclosed in the Tuvia and Garver references or other full-length L1-CAM family member proteins.

Finally, the cited references do not provide any suggestion, motivation or expectation that QFNEDGSFIGQF (SEQ ID NO: 2) or other peptides derived from the ankyrin binding domain may be used to promote neurite outgrowth, because none of the references cited by the Examiner discuss the use of competitive inhibitors of ankyrin binding to promote neurite outgrowth.

Thus, the present claims are not obvious over the cited art. Withdrawal of the obviousness rejection is respectfully requested.

CONCLUSION

Applicants request entry of the foregoing amendments and remarks in the file history of this application. In view of the above arguments, it is respectfully submitted that the present claims are now in condition for allowance and such action is earnestly solicited.

If the Examiner believes that a telephone conversation would help advance the prosecution in this case, the Examiner is respectfully requested to call the undersigned attorney at

(212) 527-7634. The Examiner is hereby authorized to charge any additional fees or credit any overpayment associated with this response to our Deposit Account No. 04-0100.

Dated: December 18, 2009

Respectfully submitted,

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EXHIBIT – A

Zhang et al.

Structural Requirements for Association of Neurofascin with Ankyrin*

(Received for publication, August 10, 1998, and in revised form, August 27, 1998)

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This paper presents the first structural analysis of the cytoplasmic domain of neurofascin, which is highly conserved among the L1CAM family of cell adhesion molecules, and describes sequence requirements for neurofascin-ankyrin interactions in living cells. The cytoplasmic domain of neurofascin dimerizes in solution, has an asymmetric shape, and exhibits a reversible temperature-dependent β -structure. Residues Ser⁵⁶–Tyr⁸¹ are necessary for ankyrin binding but do not contribute to either dimerization or formation of structure. Transfected neurofascin recruits GFP-tagged 270-kDa ankyrin_G to the plasma membrane of human embryo kidney 293 cells. Deletion mutants demonstrate that the sequence Ser⁵⁶–Tyr⁸¹ contains the major ankyrin-recruiting activity of neurofascin. Mutations of the FIGQY tyrosine (Y81H/A/E) greatly impair neurofascin-ankyrin interactions. Mutation of human L1 at the equivalent tyrosine (Y1229H) is responsible for certain cases of mental retardation (Van Camp, G., Fransen, E., Vits, L., Raes, G., and Willems, P. J. (1996) *Hum. Mutat.* 8, 391). Mutations F77A and E73Q greatly impair ankyrin binding activity, whereas mutation D74N and a triple mutation of D57N/D58N/D62N result in less loss of ankyrin binding activity. These results provide evidence for a highly specific interaction between ankyrin and neurofascin and suggest that ankyrin association with L1 is required for L1 function in humans.

L1, CHL1, neurofascin, NrCAM, and NgCAM in vertebrates and neuroglian in *Drosophila* are members of the L1CAM family of cell adhesion molecules (1, 2). These proteins possess variable ectodomains that engage in homophilic as well as heterophilic interactions and have in common a conserved cytoplasmic domain that binds to the membrane skeletal protein ankyrin (1, 3, 4). L1CAM family members are abundant in brain tissue (3, 4) and participate in diverse cellular activities including axon fasciculation, myelination, synaptogenesis, and axonal guidance (1, 5, 6). Mutations in the human L1 gene are responsible for developmental abnormalities including mental retardation and hydrocephalus (7–9).

The cytoplasmic domains of L1CAM cell adhesion molecules contain a highly conserved sequence that has been identified as a binding site for members of the ankyrin family of membrane skeletal proteins (3, 4, 10–12). Association of L1CAM molecules with ankyrin was first characterized for neurofascin, which was originally identified as a brain protein that associ-

ated with ankyrin-coupled affinity columns (3). Subsequent studies demonstrated that other L1CAM family molecules including L1, NrCAM, and neuroglian also have ankyrin binding activity in their cytoplasmic domains (4, 10, 12). The membrane binding domain of ankyrin has been demonstrated to have two distinct binding sites for neurofascin and other membrane proteins and is proposed to form lateral complexes between ion channels and cell adhesion molecules as well as to couple these proteins to the spectrin-based membrane skeleton (13–15). Physiologically relevant sites for interactions between ankyrin and L1CAM molecules include nodes of Ranvier and axon initial segments, where neurofascin and NrCAM are concentrated and co-localized with specialized isoforms of ankyrin, 270/480-kDa ankyrin_G (16). In addition, L1 and ankyrin_B are co-localized and believed to associate with each other in unmyelinated axons based on loss of L1 in premyelinated axon tracts of ankyrin_B (–/–) mice.¹

Ankyrin binding activity of neurofascin requires a highly conserved sequence from Ser⁵⁶ to Tyr⁸¹ in the cytoplasmic domain and in particular the sequence FIGQY (Phe⁷⁷–Tyr⁸¹) that is present in all L1CAM family members (11, 12). Internal deletion of these sequences abolishes ankyrin binding. In addition, the tyrosine residue in the FIGQY sequence has been identified as the major tyrosine phosphorylation site of neurofascin (11). Phosphorylation of the FIGQY tyrosine eliminates ankyrin binding of neurofascin *in vitro* and reduces coupling of neurofascin to the cytoskeleton *in vivo* (11). Inhibition of neurofascin-ankyrin interaction by tyrosine phosphorylation also results in dissociation of cell-cell adhesion mediated by homophilic interactions between neurofascin molecules expressed in cultured cells (18).

Questions remaining unanswered concern the oligomeric state and folding of the neurofascin cytoplasmic domain and the influence of tyrosine phosphorylation and mutations on these parameters. Furthermore, since previous studies on neurofascin-ankyrin interaction are largely based on *in vitro* binding assays (3, 4, 11, 14), it is not clear whether the high or low affinity interactions identified *in vitro* actually occur in living cells.

This study presents analysis of primary and secondary structural requirements for association between neurofascin and 270-kDa ankyrin_G. Ankyrin-neurofascin interactions were detected based on ability of transfected neurofascin to recruit GFP-tagged 270-kDa ankyrin_G to the plasma membrane of human embryo kidney 293 cells. Results of this assay demonstrate that the FIGQY tyrosine residue as well as certain other aromatic and negatively charged residues in the sequence Ser⁵⁶–Tyr⁸¹ are essential for the neurofascin-ankyrin interaction. Moreover, mutation of the tyrosine residue in the FIGQY sequence to histidine (Y81H) greatly impairs ankyrin binding

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¹ P. Scotland, D. Zhou, H. Benveniste, and V. Bennett, submitted for publication.

Fig. 1. The cytoplasmic domain of neurofascin is predominantly a random coil with some temperature-dependent β -structure that is unrelated to the sequence from Ser⁵⁶ to Tyr⁸¹. Circular dichroism spectra of the bacteria-expressed cytoplasmic domain of native neurofascin (A) or mutant neurofascin with deletion of the sequence from Ser⁵⁶ to Tyr⁸¹ were measured at 5, 37, and 65 °C. The dominant negative peak at about 200 nm is indicative of a random coil. The 5 °C wavelength scans were subtracted from the 65 °C scans to determine the heat-dependent β -structure, which is reflected by a positive peak at 195 nm and a negative peak at 220 nm (C). Circular dichroism experiments were performed on an Aviv 62 DS instrument. The samples were dialyzed into buffer containing 5 mM sodium phosphate and 0.5 mM sodium azide at pH 7.4. Wavelength scans were performed from 180 to 253 nm at 5, 37, and 65 °C. These results were repeated in three separate experiments. The identity of expressed neurofascin cytoplasmic domain polypeptides was confirmed by N-terminal sequencing (see "Materials and Methods").

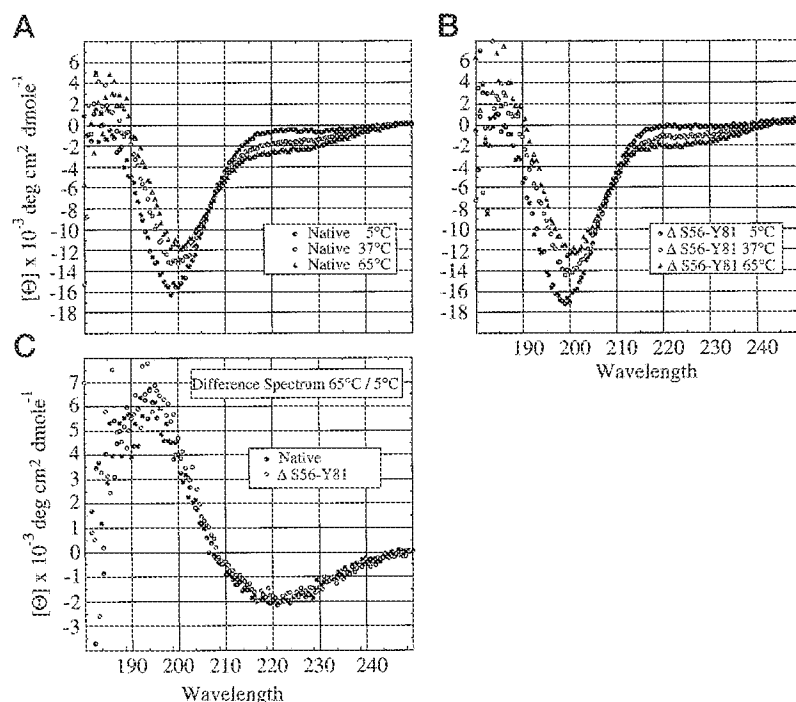


TABLE I
Summary of physical properties of the cytoplasmic domain of neurofascin

| Property | Value | |
|--|-------------------------|-------------------------|
| | Native | ΔS56-Y81 |
| Sedimentation coefficient $s_{20,w}^a$ | 1.5 | 1.6 |
| Partial specific volume, \bar{v}^b | 0.73 cm ³ /g | 0.73 cm ³ /g |
| Stokes radius, R_s^c | 3.1 nm | 3.0 nm |
| M_w , calculated ^d | 20,000 | 20,000 |
| M_w , actual ^e | 12,186 | 10,705 |
| Frictional ratio, f/f_0^f | 1.7 | 1.6 |

^a From velocity sedimentation on a Beckman ultracentrifuge.

^b Estimated from the amino acid composition.

^c The Stokes radius was determined from gel filtration on a calibrated Superose 12 column (see "Materials and Methods").

^d Calculated from the equation below and rounded to two significant figures.

$$M_r = \frac{6\pi N R_s s_{20,w}}{1 - \bar{v}\rho_{20,w}} \quad (\text{Eq. 1})$$

^e Calculated from the amino acid sequence.

^f Calculated from the following equation.

$$f/f_0 = R_s \left(\frac{4\pi N}{3M_r(\bar{v} + \delta\rho)} \right)^{1/3} \quad (\text{Eq. 2})$$

activity of neurofascin. An equivalent mutation in the human L1 gene (Y1229H) results in mental retardation and hydrocephalus (19). These results provide evidence for a highly specific interaction between ankyrin and neurofascin involving residues conserved among L1CAM family members and suggest that ankyrin association with L1 is required for L1 function in humans.

MATERIALS AND METHODS

Preparation of cDNA Constructs—Assembly of the full-length cDNA construct of 270-kDa ankyrin_G (Ank270) was achieved by ligating the first half of the membrane-binding domain, which was isolated from an adult rat brain 5'-stretch plus cDNA library (CLONTECH) by PCR² and con-

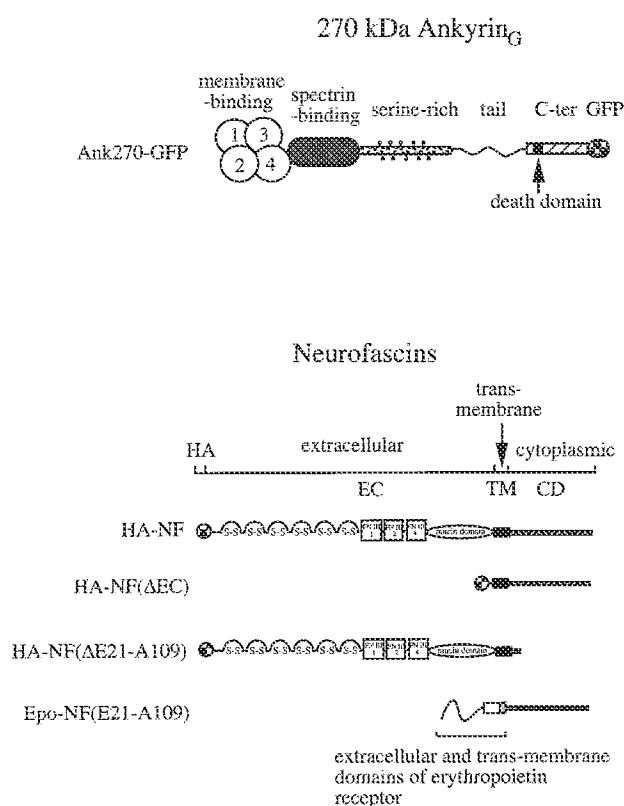


FIG. 2. Schematic diagram of cDNA constructs used in transfection experiments. Ank270-GFP represents the full-length 270-kDa ankyrin_G with a GFP tag at its C terminus. Construct HA-NF is full-length neurofascin with the HA epitope at the N terminus. Deletion of the extracellular domain results in construct HA-NF(ΔEC). Deletion of most of the cytoplasmic domain encompassing from Glu²¹ to Ala¹⁰⁹ results in construct HA-NF(ΔE21-A109). The numbers assigned to the amino acid residues are based on the published method (11), in which the first amino acid following the transmembrane domain (the lysine residue in the sequence KRSRGG; see also Fig. 2) has been assigned as the number 1 residue. Epo-NF(E21-A109) is a chimeric protein with the cytoplasmic domain of erythropoietin receptor replaced by a part of the cytoplasmic domain of neurofascin containing the sequence from Glu²¹ to Ala¹⁰⁹.

² The abbreviations used are: PCR, polymerase chain reaction; HA, hemagglutinin; NF, neurofascin; GFP, Green fluorescent protein.

firmed by DNA sequencing, with the construct M-Sb-Sr-TC (20) through *EcoRI*–*NsiI* sites. The C-terminal domain of 270-kDa ankyrin_C was PCR-amplified and introduced into the *Sall* site of the pEGFP-N1 vector (CLONTECH), while keeping in frame with the downstream EGFP protein. The resultant construct is named Ank-Ct. The full-length cDNA construct of 270-kDa ankyrin_C with GFP tagged at the C terminus (Ank270-GFP; Fig. 2) was prepared by ligating the *EcoRI*–*EcoRV* fragment of construct Ank270 into the *EcoRI*–*EcoRV* sites of construct Ank-Ct. The resultant construct (Ank270-GFP; Fig. 2) is driven by the cytomegalovirus promoter. The full-length rat neurofascin cDNA with an HA epitope at the N terminus (11) was cut out by *HindIII*–*NotI* digestion of pBluescript KS vector (Stratagene) and subcloned into the corresponding sites of pEGFP-N1 vector (CLONTECH). The resultant construct (HA-NF; Fig. 1) does not contain the EGFP sequence and is driven by a cytomegalovirus promoter. All other neurofascin constructs were prepared from construct HA-NF unless indicated. The cytoplasmic domain-deleted neurofascin HA-NF(ΔE21–A109) was made by replacing the *ScaI*–*NotI* fragment of construct HA-NF with a PCR-amplified fragment containing the sequence of the *ScaI*–*ApaI* fragment with a stop codon following the *ApaI* site (Fig. 2). The extracellular domain-truncated neurofascin (HA-NF(ΔEC); Fig. 2) was prepared through two steps. First, the 5'-untranslated region of neurofascin, the start codon, the signal peptide, and HA tag were PCR-amplified and subcloned into the *BglII*–*HindIII* sites of the pEGFP-N1 vector. Then the transmembrane and cytoplasmic domains were introduced by PCR into the *HindIII*–*NotI* sites of the first step construct. The chimeric protein with the cytoplasmic domain of erythropoietin receptor replaced by a part of the cytoplasmic domain of neurofascin (Epo-NF(E21–A109); Fig. 2) was prepared by replacing the *HindIII*–*ApaI* fragment of HA-NF with a PCR-amplified sequence containing the extracellular and transmembrane domains of the erythropoietin receptor, which was kindly provided by Dr. Harvey Lodish (MIT).

Neurofascin constructs with deletions from the C terminus of the cytoplasmic domain (HA-NF(ΔT82–A109), HA-NF(ΔY81–A109), and HA-NF(ΔF77–A109); see Fig. 5) were prepared by replacing the *ApaI*–*NotI* fragment of HA-NF with the PCR-amplified sequences encompassing from Gly¹⁹ to various positions inside the cytoplasmic domain (Tyr⁸¹, Glu⁸⁰, and Ser⁷⁶, respectively). The internal deletions (HA-NF(ΔF77–Y81), HA-NF(ΔQ70–Y81), HA-NF(ΔS56–Y81); see Fig. 5) were prepared as described (11). Other internal deletions (HA-NF(ΔE21–E55), HA-NF(ΔE21–G64), and HA-NF(ΔE21–Q70)) were constructed by replacing the *ApaI*–*NotI* fragment with the PCR-amplified sequences that start from various corresponding sites in the cytoplasmic domain (Ser⁶⁶, Glu⁶⁵, and Phe⁷¹, respectively) and end at the stop codon of the wild type neurofascin in construct HA-NF (Fig. 5).

Point mutations of the cytoplasmic domain of neurofascin were prepared using the QuickChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing (Fig. 5). The *ApaI*–*NotI* fragment of HA-NF was subcloned into pBluescript vector, in which site-directed mutagenesis was carried out. The confirmed sequences with single amino acid mutations were subcloned back into the exact sites of construct HA-NF from which they had been cut out.

cDNA Transfection and Immunofluorescence.—Human kidney 293 cells were cultured in 10% fetal bovine serum in Dulbecco's modified Eagle's medium and transfected with LipofectAMINE in Opti-MEM serum-free medium following the manufacturer's protocol (Life Technologies, Inc.). The amount of each species of co-transfected cDNAs was adjusted to a 1:1 molar ratio. In most cases, 1 μg of neurofascin cDNA or its mutants and 1.5 μg of 270-kDa ankyrin_C cDNA were applied for each co-transfection experiment in a 35-mm culture dish. All co-transfection experiments and subsequent immunofluorescence experiments were performed under the same conditions.

For immunofluorescence, transfected 293 cells were fixed in 2% paraformaldehyde for 10 min and then incubated with blocking buffer (10% normal goat serum and 2% bovine serum albumin in phosphate-buffered saline) for another 5 min before applying the primary antibody against HA epitope (Babco). After 3 h of incubation with the primary antibody, cells were washed three times with phosphate-buffered saline and subjected to secondary stain using tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse antibody for 1 h. All immunostaining procedures were performed at room temperature. In the case of co-transfection of Ank270-GFP and Epo-NF(E21–A109), 0.1% Triton X-100 was included in the blocking buffer, and the primary stain was an antibody specific for the FICQY sequence in the cytoplasmic domain of neurofascin. All immunofluorescence experiments were recorded using a Zeiss LSM 410 confocal microscope. Experiments were replicated at least three times.

Determination of Physical Properties of the Cytoplasmic Domain of Neurofascin.—DNA constructs of the neurofascin cytoplasmic domain

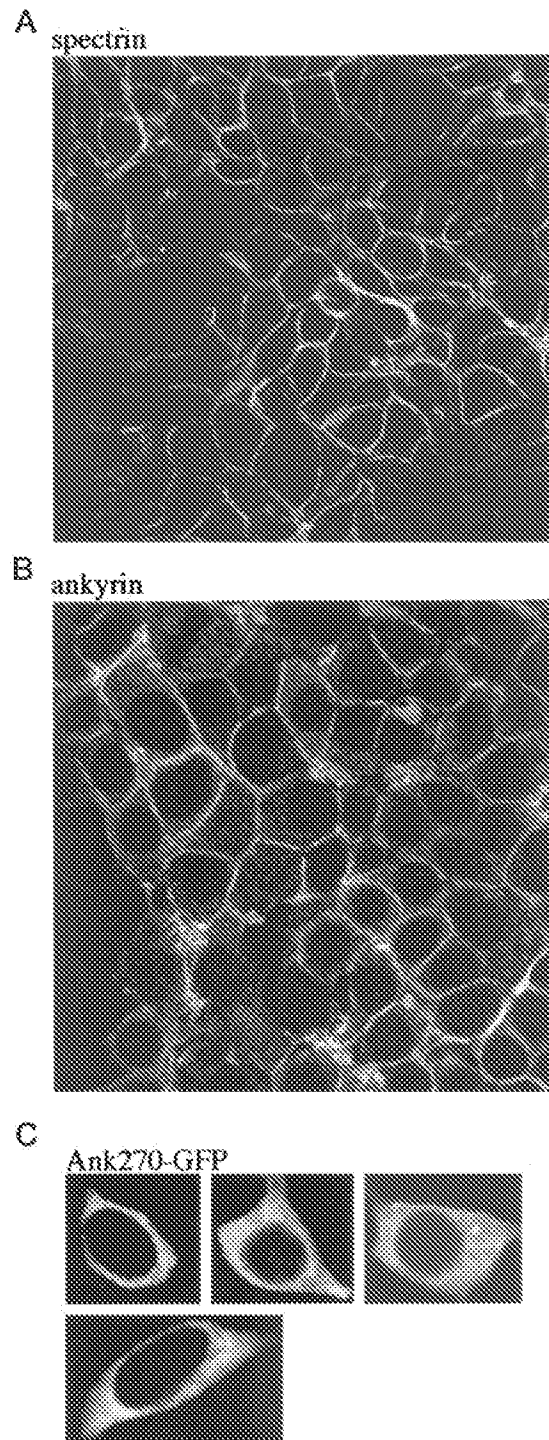


FIG. 3. Endogenous spectrin of 293 cells is insufficient to recruit transfected ankyrin to the plasma membrane. A, immunostaining of endogenous spectrin in 293 cells, which is predominantly localized at the plasma membrane. B, immunostaining of endogenous ankyrin, which is also concentrated at the plasma membrane. C, transfected 270-kDa ankyrin_C (Ank270-GFP) visualized by GFP signal is distributed throughout the cytoplasm of 293 cells.

were amplified by PCR using 186-kDa rat neurofascin cDNA as a template (3) with added 5' *NcoI* and a 3' *XhoI* restriction sites for subcloning. The PCR products were restricted and ligated into a Pet vector with a C-terminal histidine tag (Novagen Pet 28b(+)). Cytoplasmic domain lacking the histidine tag was also expressed and exhibited the same properties as His tag constructs. Plasmids were transformed into BL21 DE3 pLysS bacteria and expressed with isopropyl-1-thio-β-D-galactopyranoside induction. Expressed proteins with histidine tags were purified from the soluble fraction of lysed bacteria using a nickel-

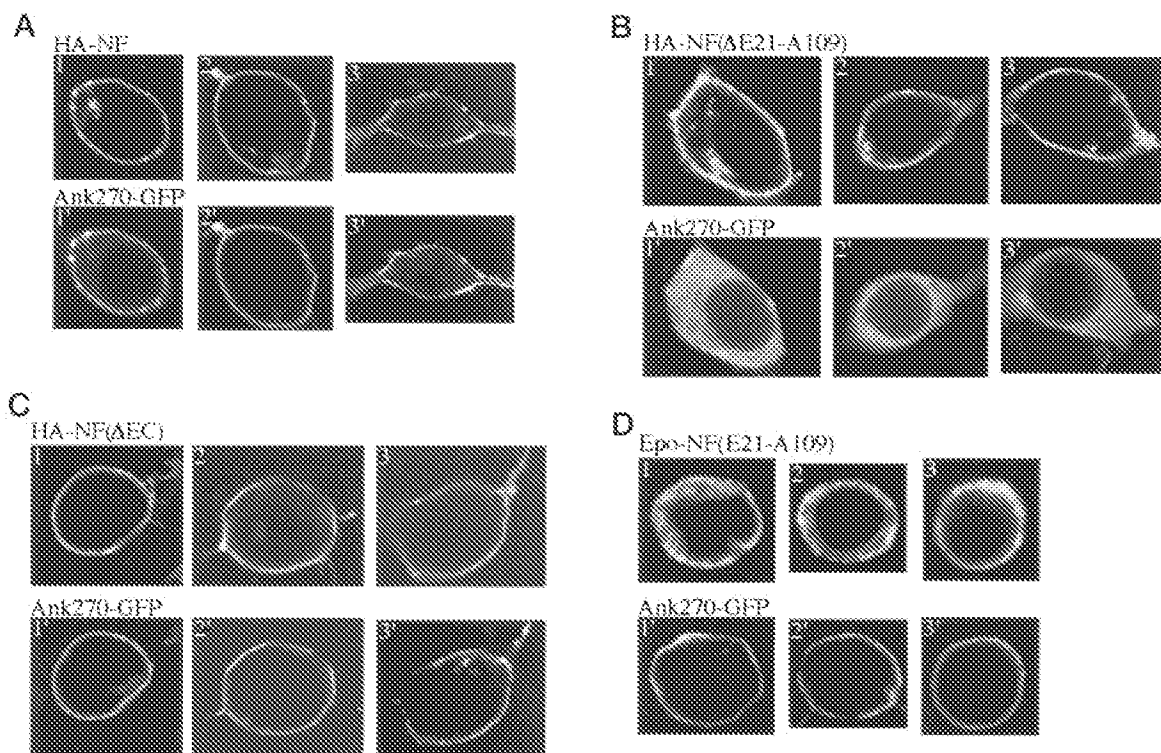


Fig. 4. The cytoplasmic domain of neurofascin is sufficient and necessary for recruitment of co-transfected ankyrin to the plasma membrane. A, double labeling of co-transfection of neurofascin (HA-NF) and 270-kDa ankyrin_G (Ank270-GFP) in 293 cells. Transfected ankyrin (Ank270-GFP) is recruited to the plasma membrane. B, double labeling of co-transfection of the cytoplasmic domain-deleted neurofascin (HA-NF(ΔE21-A109)) and Ank270-GFP. Ank270-GFP is predominantly localized in the cytoplasm instead of at the plasma membrane. C, double labeling of co-transfection of extracellular domain-truncated neurofascin (HA-NF(ΔEC)) and Ank270-GFP. Ank270-GFP is associated with the plasma membrane. D, co-transfection of chimeric protein Epo-NF(E21-A109) and Ank270-GFP. Epo-NF(E21-A109) recruits Ank270-GFP to the plasma membrane, although the majority of the expressed Epo-NF(E21-A109) is localized in the cytoplasm of transfected cells. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 in A, B, and C are immunostaining of the HA epitope of neurofascin. 1', 2', and 3' in D are immunostaining of the cytoplasmic domain of neurofascin using an antibody specific for the FIGQY sequence. 1, 2, and 3 in D are immunostaining of the cytoplasmic domain of neurofascin using an antibody specific for the FIGQY sequence. These results as well as those described below were replicated in at least three separate experiments.

nitrilotriacetic acid-agarose affinity column (Qiagen). The eluate from the nickel column was then dialyzed and applied to a Mono S high pressure liquid chromatography ion exchanger column, which was subsequently eluted with a sodium chloride salt gradient. Proteins lacking a histidine tag were isolated using Mono Q ion exchange chromatography and gel filtration on Superose 12 in addition to Mono S ion exchange chromatography. The identity of purified polypeptides was established by N-terminal sequencing following transfer to polyvinylidene difluoride paper (3, 13). The purity of polypeptides was at least 90% based on SDS-polyacrylamide gel electrophoresis.

The Stokes radii (R_s) of cytoplasmic domains were estimated by gel filtration on a Superose 12 column equilibrated with 10 mM sodium phosphate, 100 mM NaCl, 0.5 mM dithiothreitol, 1 mM NaN_3 , pH 7.4, and calibrated with the following protein standards: ferritin ($R_s = 6.1$ nm), catalase ($R_s = 5.2$ nm), bovine serum albumin ($R_s = 3.5$ nm), ovalbumin ($R_s = 3.05$ nm), and cytochrome *c* ($R_s = 2$ nm). The sedimentation coefficients were determined by sedimentation equilibrium on a Beckman XL-A Optima analytical centrifuge, and the sedimentation patterns were analyzed by the Ideal 1 software program from Beckman. The measurements of Table I were repeated three times with comparable results. Values are presented for a single representative experiment.

RESULTS

Secondary Structure and Oligomeric State of the Cytoplasmic Domain of Neurofascin.—The secondary structure of the cytoplasmic domain of native neurofascin expressed in bacteria was evaluated by CD spectroscopy (Fig. 1). At 5 °C, the dominant negative peak at 200 nm in the CD spectrum (Fig. 1A) suggested the native cytoplasmic domain is predominantly a random coil (21). Hydrodynamic measurements of the Stokes radius ($R_s = 3.1$ nm) and the frictional ratio ($f/f_0 = 1.7$) also indicate an extended conformation of the cytoplasmic domain of

native neurofascin (Table I). At higher temperatures (37 and 65 °C), however, there is evidence of structure, as demonstrated by the reduction of absorbance above 210 nm at higher temperatures (Fig. 1A). Difference spectra between 65 and 5 °C revealed a curve consistent with β -structure with a positive peak at 195 nm as well as a negative peak at 220 nm (Fig. 1C). The appearance of β -structure is fully reversible upon lowering the temperature (data not shown). This temperature dependence implies hydrophobic interactions involved in stabilizing β -structure in the cytoplasmic domain of neurofascin (21).

The sequence Ser⁵⁶–Tyr⁸¹ is necessary for ankyrin binding to intact neurofascin expressed in neuroblastoma cells (11). The contribution of residues Ser⁵⁶–Tyr⁸¹ to the secondary structure of the cytoplasmic domain of neurofascin expressed in bacteria was evaluated by measuring the CD spectrum of the cytoplasmic domain with internal deletion of Ser⁵⁶–Tyr⁸¹ (Fig. 1B). The appearance of a positive peak at 195 nm and a negative peak at 220 nm in the difference spectrum, which is almost identical to that of native neurofascin, indicates that the sequence Ser⁵⁶–Tyr⁸¹ does not contribute to formation of the temperature-dependent secondary structure.

The cytoplasmic domain of neurofascin exists as a dimer or in a monomer-dimer equilibrium, based on molecular weight determined by sedimentation equilibrium measurements (Table I). The cytoplasmic domain with internal deletion of Ser⁵⁶–Tyr⁸¹ also behaves as a dimer (Table I). Sedimentation measurements were performed at 25 °C, which is a condition with minimal temperature-induced secondary structure detected by CD spectroscopy. Therefore, assembly of neurofascin cytoplas-

| The sequence of the cytoplasmic domain of neurofascin and its mutants | | | | | | | | | | membrane- recruitment of ankyrin | | | | | | | | | | | | | | | | | | | | | | |
|---|------------------|------------|-------|--------|-----|--------|---------|----------------|--------|-------------------------------------|-------|-------|----|-----|------|------|--------|---------------------|-------------------------------|------|-------------------|--------------------|-------------------------------|------|--|--|--|--|--|--|-------------------------------|---|
| | 21 | 31 | 41 | 51 | 61 | 71 | 81 | 91 | 101 | | | | | | | | | | | | | | | | | | | | | | | |
| Consensus: | --D-- | DEG-FEYSD | --K-- | S----- | K-- | SDDS-- | DYG---- | QFNEDGSFIGQY-- | KK---- | | | | | | | | | | | | | | | | | | | | | | | |
| | : | :::1:::2:: | : | 1 | | 1211 | 11 | :::11111111:: | :: | | | | | | | | | | | | | | | | | | | | | | | |
| HA-NF: | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGSFIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | ++++ | | | | | | | | | | | | |
| HA-NF(ΔE21-A109): GP | | | | | | | | | | | | | | | | | | | | | - | | | | | | | | | | | |
| HA-NF(ΔT82-A109): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGSFIGQY | | ++++ | | | | | | | | | | | | |
| HA-NF(ΔY81-A109): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGSFIGQ | | + | | | | | | | | | | | | |
| HA-NF(ΔF77-A109): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGS | | - | | | | | | | | | | | | |
| HA-NF(ΔF77-Y81): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGS | TVRKDKKEETEGNESSEATSPVNAIYSLA | - | | | | | | | | | | | | |
| HA-NF(ΔQ70-Y81): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEG | TVRKDKKEETEGNESSEATSPVNAIYSLA | - | | | | | | | | | | | | |
| HA-NF(ΔS56-Y81): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEG | TVRKDKKEETEGNESSEATSPVNAIYSLA | - | | | | | | | | | | | | |
| HA-NF(ΔE21-E55): GP | | | | | | | | | | | SDDS | | | | | | | | | | DSLVDY | GEGGEGQFNEDGSFIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | ++++ | | | | | | | | |
| HA-NF(ΔE21-G64): GP | | | | | | | | | | | | | | | | | | | | | EGGEGQFNEDGSFIGQY | | | | | | | | | | TVRKDKKEETEGNESSEATSPVNAIYSLA | + |
| HA-NF(ΔE21-Q70): GP | | | | | | | | | | | | | | | | | | | | | FNEDGSFIGQY | | | | | | | | | | TVRKDKKEETEGNESSEATSPVNAIYSLA | + |
| HA-NF(Y81F): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGSFIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | ++++ | | | | | | | | | | | | |
| HA-NF(Y81A): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGSFIGQA | TVRKDKKEETEGNESSEATSPVNAIYSLA | - | | | | | | | | | | | | |
| HA-NF(Y81E): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGSFIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | + | | | | | | | | | | | | |
| HA-NF(Y81H): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGSFIGQH | TVRKDKKEETEGNESSEATSPVNAIYSLA | + | | | | | | | | | | | | |
| HA-NF(F77A): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGSAIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | + | | | | | | | | | | | | |
| HA-NF(F71A): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQANEDGSFIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | ++ | | | | | | | | | | | | |
| HA-NF(F71L): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQLNEDGSFIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | ++ | | | | | | | | | | | | |
| HA-NF(E73Q): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNQDGSFIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | + | | | | | | | | | | | | |
| HA-NF(D74N): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNENGSGFIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | +++ | | | | | | | | | | | | |
| HA-NF(D57,S8,G2N): | GPEDPKEEDGSFDYSD | | | | | | | | | | EDNKP | LQGSQ | TS | LDG | TIKQ | QESD | DSLVDY | GEGGEGQFNEDGSFIGQY | TVRKDKKEETEGNESSEATSPVNAIYSLA | ++++ | | | | | | | | | | | | |

Fig. 5. Summary of effects of different mutations in the cytoplasmic domain of neurofascin on membrane recruitment of co-transfected 270-kDa ankyrin_G. Numbers for the amino acid residues in the cytoplasmic domain of neurofascin are assigned based on the method of Carver *et al.* (11), in which the first amino acid following the transmembrane domain (the lysine residue in the sequence KRSRGG) has been assigned as the number 1 residue (not shown in the displayed sequence). The COOH terminus residue is Ala¹⁰⁹. The consensus sequence is conserved (indicated by dots) among L1CAM molecules of vertebrate as well as invertebrate animals. A boldface 1 denotes an amino acid residue that is identical among L1CAM members. Evaluation of neurofascin-ankyrin interaction is primarily based on the extent of transfected 270-kDa ankyrin_G (Ank270-GFP) being recruited to the plasma membrane by co-transfected neurofascins (see text). The intensity ratio between the GFP signals at the plasma membrane and inside the cytoplasm (*M/C*) is used as an approximate scale to measure the affinity of the association of 270-kDa ankyrin_G with co-transfected neurofascins. +++++, *M/C* > 5; +++, 2 < *M/C* < 5; ++, 1.5 < *M/C* < 2; +, 1.1 < *M/C* < 1.5; -, *M/C* < 1.1.

mic domains into dimers does not require the thermally induced folding detected in Fig. 1. The cytoplasmic domain contains only minimal predicted coiled-coil α -helix, which is consistent with CD data but leaves unexplained the basis for dimerization.

These data demonstrate that, although the cytoplasmic domain of neurofascin is predominantly a random coil, it is capable of forming a dimer and β -structure at physiological temperatures. However, there is no evidence that the sequence from Ser⁵⁶ to Tyr⁸¹, which is essential for ankyrin binding to full-length neurofascin (11), contributes to dimerization or to formation of β -structure. The sequence Ser⁵⁶-Tyr⁸¹ thus has little secondary structure, although this segment could be configured as a loop or turn that is not easily detected by CD spectroscopy.

Development of an Assay to Evaluate Neurofascin-Ankyrin Interactions in Living Cells.—To evaluate interactions between ankyrin and neurofascin in cells, we developed an assay system based on co-transfection of GFP-tagged 270-kDa ankyrin_G and HA-tagged neurofascin (Fig. 2) into human embryonic kidney cells (293 cells). Endogenous spectrin and endogenous ankyrin were concentrated at the plasma membrane of 293 cells (Fig. 3). However, transfected GFP-tagged 270-kDa ankyrin_G (Ank270-GFP) was distributed throughout the cytoplasm (Fig. 3). GFP-tagged ankyrin was recruited to the plasma membrane when co-transfected with neurofascin (HA-NF) (Fig. 4A). The ability of neurofascin to recruit ankyrin to the plasma membrane did not require the extracellular domain of neurofascin. Deletion of the extracellular domain (HA-NF(ΔEC)) had little effect on recruitment of co-transfected ankyrin to the plasma membrane (Fig. 4C).

The cytoplasmic domain of neurofascin was necessary and sufficient to provide effective membrane-binding sites for recruitment of transfected ankyrin to the plasma membrane (Fig. 4, B and D). Deletion of a major portion of the cytoplasmic domain of neurofascin extending from Glu²¹ to the C terminus residue Ala¹⁰⁹ (HA-NF(ΔE21-A109); Fig. 5) abolished ankyrin recruitment to the plasma membrane (Fig. 4B). The numbers

assigned to the amino acid residues in the cytoplasmic domain of neurofascin are based on the convention where the first amino acid following the transmembrane domain (the lysine residue in the sequence KRSRGG; see Fig. 5) is residue 1 (11). The addition of the sequence from Glu²¹ to Ala¹⁰⁹ to the cytoplasmic domain-deleted erythropoietin receptor resulted in a chimeric protein (Epo-NF(E21-A109)) with full activity in recruiting co-transfected ankyrin to the plasma membrane (Fig. 4D). These data indicate that the sequence from Glu²¹ to Ala¹⁰⁹ of the cytoplasmic domain of neurofascin determines activity in binding to ankyrin.

The Sequence Ser⁵⁶-Tyr⁸¹ Contains the Major Ankyrin-binding Site of Neurofascin.—The *in vivo* assay described above was used to evaluate the effect on ankyrin binding of various internal deletions in the cytoplasmic domain of neurofascin. Internal deletion from Ser⁵⁶ to Tyr⁸¹ (HA-NF(ΔS56-Y81)) (Fig. 5) abolished recruitment of co-transfected ankyrin to the plasma membrane in the *in vivo* assay (Fig. 6A), as observed previously in direct binding assays (11). Two other mutants with shorter internal deletions, one from Glu⁷⁰ to Tyr⁸¹ (HA-NF(ΔQ70-Y81)) and the other from Phe⁷⁷ to Tyr⁸¹ (HA-NF(ΔF77-Y81)) (Fig. 5), also eliminated the ability of neurofascin to recruit co-transfected ankyrin to the plasma membrane (Fig. 6, B and C, respectively). Neurofascin with internal deletion of Phe⁷⁷-Tyr⁸¹ retained about 20% ankyrin binding activity compared with the native protein in *in vitro* binding assays (11). However, the cell assay developed in this study failed to detect this residual ankyrin binding activity (Fig. 6C).

Residues in the cytoplasmic domain of neurofascin preceding Ser⁵⁶ and following Tyr⁸¹ do not contribute significantly to ankyrin binding activity. As demonstrated above using constructs Epo-NF(E21-A109) and HA-NF(ΔE21-A109) (Fig. 5), the sequence before Glu²¹ that includes the extracellular and transmembrane domains and part of the cytoplasmic domain (K1-P20) was neither necessary (Fig. 4D) nor sufficient (Fig. 4B) for recruitment of ankyrin to the plasma membrane. A neurofascin mutant with an internal deletion of the sequence

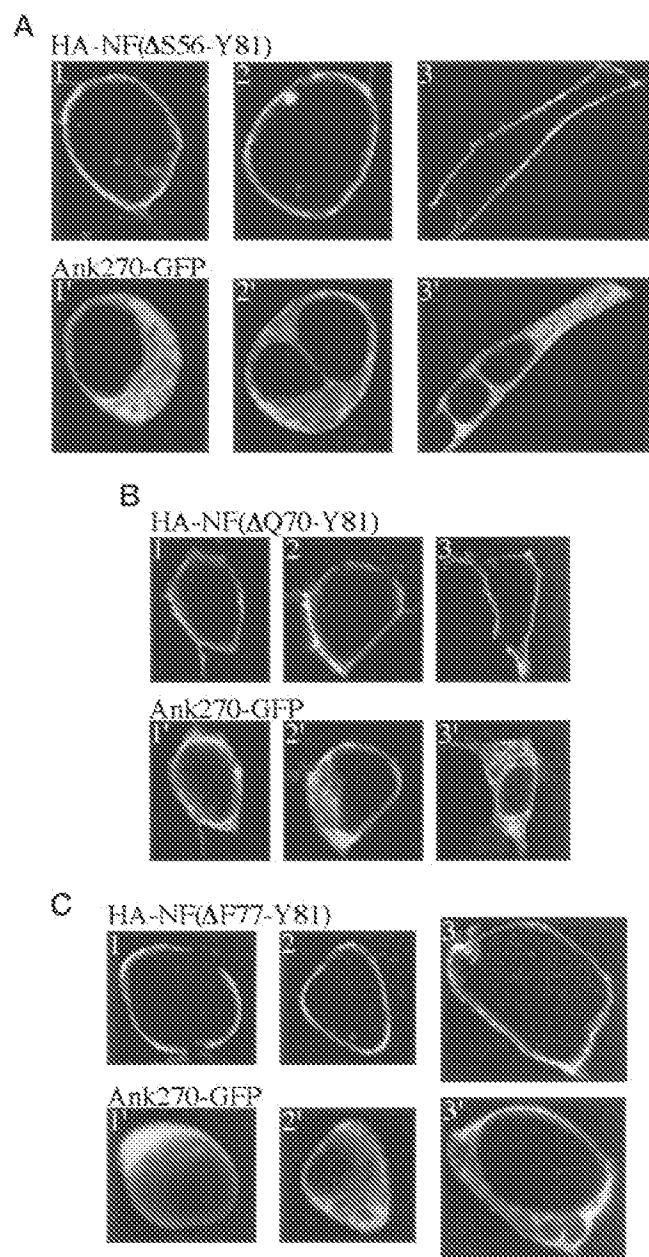


FIG. 6. The sequence from Ser⁵⁶ to Tyr⁸¹ in the cytoplasmic domain of transfected neurofascin is necessary for recruitment of co-transfected ankyrin to the plasma membrane. Internal deletion of the sequence from Ser⁵⁶ to Tyr⁸¹ (HA-NF(ΔS56-Y81)) (A) and internal deletion from Glu⁷⁰ to Tyr⁸¹ (HA-NF(ΔQ70-Y81)) (B) abolish the ability of the mutant neurofascins to recruit co-transfected Ank270-GFP to the plasma membrane. C, internal deletion of the FIGQY sequence (HA-NF(ΔF77-Y81)) also eliminate the ability of the mutant neurofascin to recruit Ank270-GFP to the plasma membrane. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 are immunostaining of the HA epitope of mutant neurofascins. 1', 2', and 3' are the GFP fluorescence of co-transfected Ank270-GFP.

from Glu²¹ to Glu⁵⁵ (HA-NA(ΔE21-E55)) retained full activity to recruit transfected ankyrin to the plasma membrane (Fig. 7A). Deletion of the sequence following Tyr⁸¹ (HA-NA(ΔT82-A109)) also exerted little effect on recruitment of ankyrin to the plasma membrane (Fig. 8A).

While residues outside of the critical Ser⁵⁶–Tyr⁸¹ stretch are not required for recruitment of ankyrin to the plasma membrane, sequences within Ser⁵⁶–Tyr⁸¹ are essential for activity. Internal deletions extending from Glu²¹ to Gly⁶⁴ (HA-

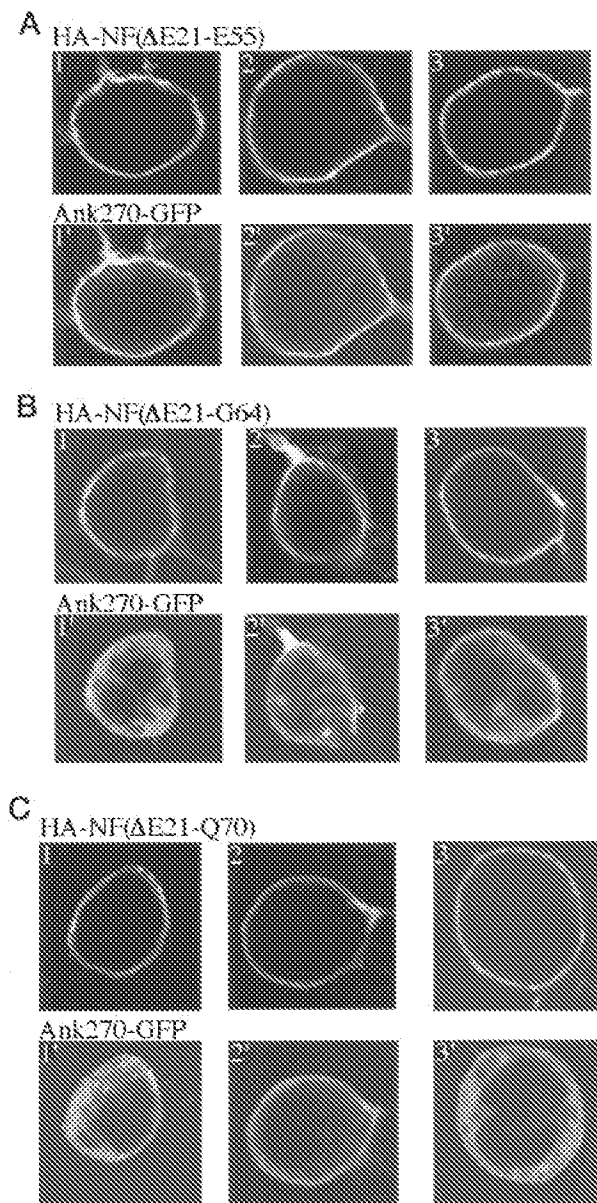


FIG. 7. The sequence following Ser⁵⁶ is sufficient for recruitment of co-transfected ankyrin to the plasma membrane. A, neurofascin mutant with internal deletion from Glu²¹ to Glu⁵⁵ (HA-NF(ΔE21-E55)) retains full ability to recruit co-transfected Ank270-GFP to the plasma membrane. B and C, internal deletions from Glu²¹ to Gly⁶⁴ (HA-NF(ΔE21-G64)) and from Glu²¹ to Glu⁷⁰ (HA-NF(ΔE21-Q70)), respectively, abolish ankyrin-recruiting activity. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 are immunostaining of the HA epitope of mutant neurofascins. 1', 2', and 3' are the GFP fluorescence of co-transfected Ank270-GFP.

NA(ΔE21-G64)) or from Glu²¹ to Glu⁷⁰ (HA-NA(ΔE21-Q70)) (Fig. 5) greatly impair ankyrin-recruiting activity (Fig. 7, B and C, respectively). Deletion of residues from Phe⁷⁷ to Ala¹⁰⁹ (HA-NA(ΔF77-A109)) (Fig. 5) totally abolished ankyrin recruitment to the plasma membrane (Fig. 8C). Taken together, the above data indicate that the sequence from Ser⁵⁶ to Tyr⁸¹ contains the major ankyrin-binding site in the cytoplasmic domain of neurofascin.

The FIGQY Tyrosine (Tyr⁸¹) Is Essential for Neurofascin-Ankyrin Interactions—The neurofascin mutant with deletion of the sequence following Tyr⁸¹ (HA-NA(ΔT82-A109); Fig. 5) retains full activity in recruitment of transfected ankyrin to the plasma membrane (Fig. 8A). However, if the deletion extends one additional residue to include the tyrosine residue Tyr⁸¹

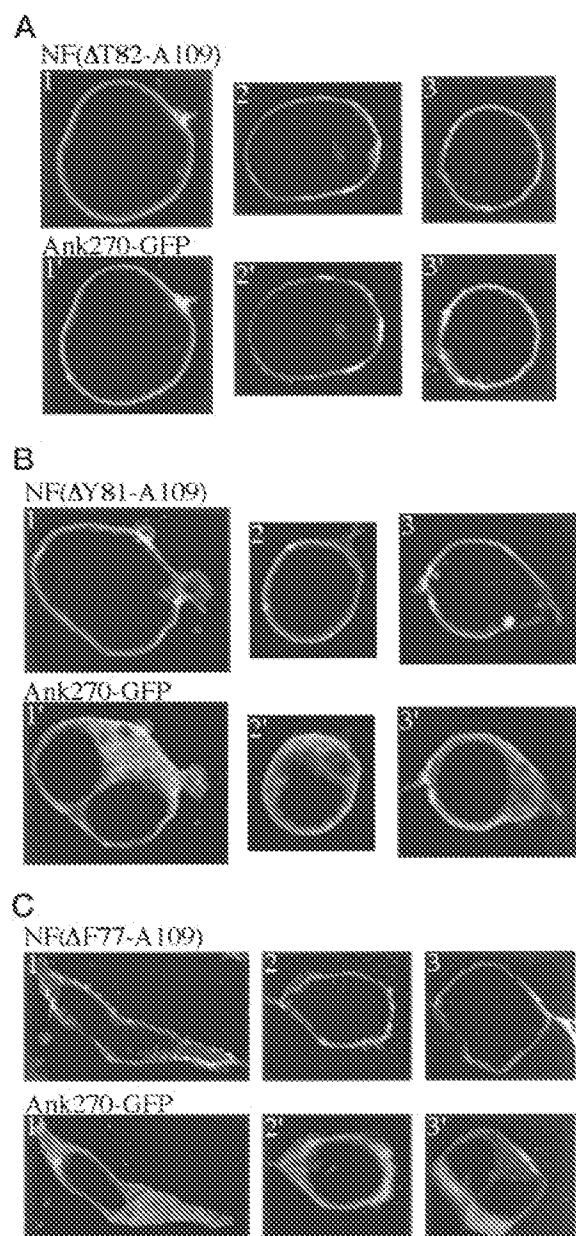


Fig. 8. The tyrosine residue Tyr⁸¹ in the FIGQY sequence is essential for neurofascin-ankyrin interactions. A, neurofascin mutant with deletion of the sequence following Tyr⁸¹ (HA-NF(ΔT82-A109)) retains the full ankyrin recruitment activity. B, deletion of Tyr⁸¹ along with the sequence following it (HA-NF(ΔY81-A109)) greatly impairs ankyrin-recruitment activity. C, deletion of the sequence following Phe⁷⁷ (HA-NF(ΔF77-A109)) totally eliminates ankyrin-recruiting activity. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 are immunostaining of the HA epitope of mutant neurofascins. 1', 2', and 3' are the GFP fluorescence of co-transfected Ank270-GFP.

(HA-NA(ΔY81-A109); Fig. 5), recruitment of transfected ankyrin to the plasma membrane is almost abolished (Fig. 8B). These experiments demonstrated a critical role for the FIGQY tyrosine in neurofascin-ankyrin interactions.

Mutations of Tyr⁸¹ were used to further evaluate the structural requirements at the FIGQY tyrosine site. The mutation Y81F (HA-NF(Y81F)) retains full ankyrin-recruiting activity (Fig. 9A), suggesting the hydroxyl group of the tyrosine residue is not directly involved in binding. Previous studies have demonstrated that phosphorylation of Tyr⁸¹ at this hydroxyl group abolishes ankyrin binding (11, 18). Mutation of Tyr⁸¹ to glutamic acid (HA-NF(Y81E)) mimics phosphorylation by intro-

ducing negative charges and greatly impairs recruitment of ankyrin to the plasma membrane (Fig. 9C). Mutation of Tyr⁸¹ to alanine (HA-NF(Y81A)) totally abolishes ankyrin-recruitment (Fig. 9B), suggesting that the aromatic ring of Tyr⁸¹ also is involved in neurofascin-ankyrin interaction.

The clinical importance of the tyrosine residue in the FIGQY sequence is emphasized by the finding that a mutation in the cytoplasmic domain of human L1 molecule with the corresponding tyrosine residue changed to histidine (Y1229H) is responsible for the development of mental retardation and hydrocephalus (19). An equivalent mutation in neurofascin (Y81H) was prepared and evaluated for its activity to recruit co-transfected ankyrin to the plasma membrane. Mutation of Tyr⁸¹ to histidine greatly impairs ankyrin recruitment activity in 293 cells (Fig. 9D). This result suggests that disruption of the interaction between L1 and ankyrin may be the molecular basis for symptoms of patients with the L1 Y1229H mutation.

Interaction between Neurofascin and Ankyrin Involves Conserved Aromatic and Negatively Charged Residues within the Gln⁷⁰-Tyr⁸¹ Stretch—Other aromatic residues in addition to Tyr⁸¹ in the highly conserved Q70FNEDGSFIGQY81 sequence also participate in association between neurofascin and ankyrin. F77A (HA-NF(F77A)) and F71A (HA-NF(F71A)) (Fig. 5) mutations both impair ankyrin recruitment to the plasma membrane (Fig. 10, A and B, respectively). Moreover, mutation F71L (HA-NF(F71L)), compared with mutation F71A, does not significantly improve ankyrin recruitment to the plasma membrane (Fig. 10C), suggesting that the function of the aromatic group in neurofascin-ankyrin interactions cannot be replaced by a hydrophobic side chain.

Electrostatic interactions between neurofascin and ankyrin have been inferred from *in vitro* binding assays based on sensitivity to salt (13, 14). Neutralizing a negative charge by mutation of Glu⁷³ to glutamine (HA-NF(E73Q)) abolished the ankyrin-recruiting activity of the mutant neurofascin (Fig. 11A). However, mutation of Asp⁷⁴ to asparagine (HA-NF(D74N)) had much less effect on neurofascin-ankyrin association (Fig. 11B). Changing all three aspartic acid residues at Asp⁵⁷, Asp⁵⁸, and Asp⁶² to asparagine (HA-NF(D57,58,62N)) had little effect on recruiting ankyrin to the plasma membrane (Fig. 11C). These data (summarized in Fig. 5) indicate that electrostatic interactions between neurofascin and ankyrin are confined to specific charged residues and provide evidence for a high degree of specificity in the interaction between neurofascin and ankyrin.

DISCUSSION

This paper presents the first structural analysis of the conserved cytoplasmic domain of the L1CAM family of cell adhesion molecules and provides a detailed structural and functional analysis of neurofascin-ankyrin interactions in living cells. The cytoplasmic domain of neurofascin dimerizes in solution, has an asymmetric shape, and exhibits a reversible temperature-dependent β -structure. The sequence from Ser⁵⁶ to Tyr⁸¹, which is necessary for ankyrin binding, does not contribute to either dimerization or formation of structure in the cytoplasmic domain of neurofascin. A qualitative assay for evaluation of neurofascin-ankyrin interactions in living cells has been developed. Using this assay, we confirmed results of previous *in vitro* binding assays in living cells (11) and provided additional evidence that the sequence from Ser⁵⁶ to Tyr⁸¹ contains the major ankyrin-binding site. The FIGQY tyrosine (Tyr⁸¹), which is the site for tyrosine phosphorylation that abolishes ankyrin binding (11), has been demonstrated to be critical for neurofascin-ankyrin interactions. Mutation of the FIGQY tyrosine to histidine greatly impairs neurofascin-ankyrin interactions. An equivalent mutation in the human L1

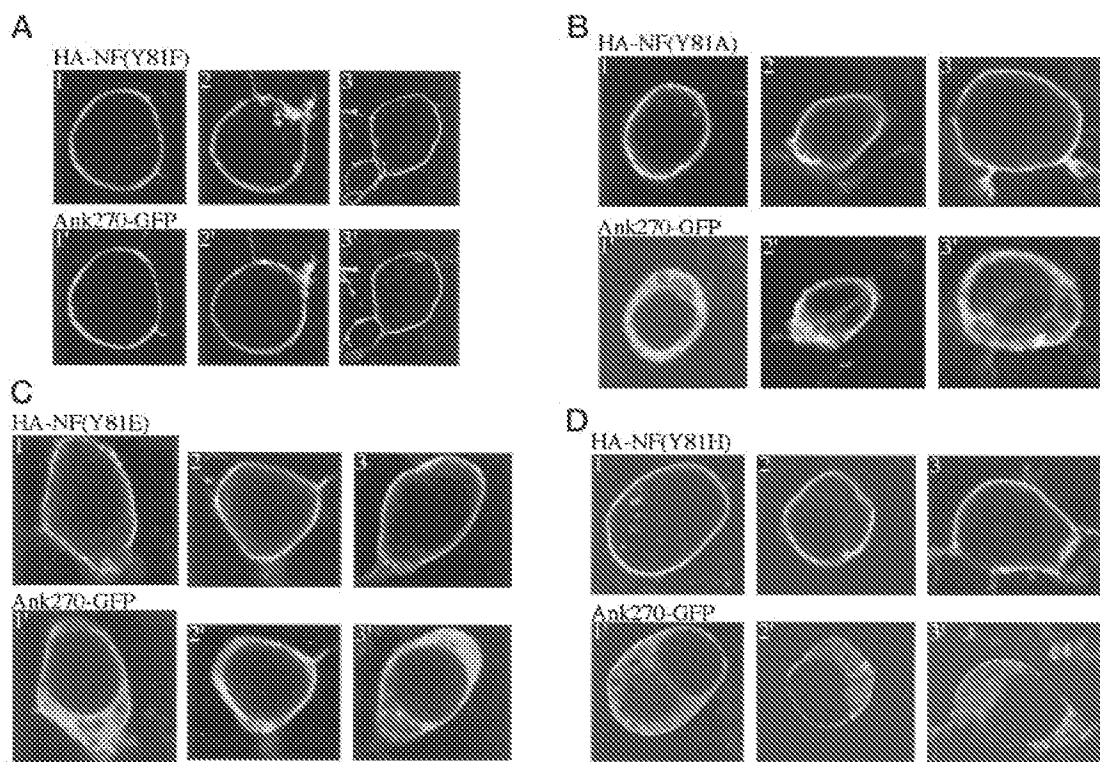


Fig. 9. Site mutations of Tyr⁵¹ impair neurofascin-ankyrin interactions. A, site mutation Y81F retains full ankyrin-recruiting activity. B, site mutation Y81A abolishes recruitment of co-transfected Ank270-GFP to the plasma membrane. Mutation Y81E (C) and mutation Y81H (D) greatly impair membrane-recruitment of co-transfected Ank270-GFP. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 are immunostaining of the HA epitope of mutant neurofascins. 1', 2', and 3' are the GFP fluorescence of co-transfected Ank270-GFP.

molecule (Y1229H) is responsible for certain cases of hydrocephalus and mental retardation (19). Since L1 and neurofascin share a conserved cytoplasmic binding site for ankyrin (4, 11), disruption of L1-ankyrin interactions may be the molecular basis for pathology due to this L1 mutation (Y1229H). Other conserved aromatic and negatively charged residues in the sequence Ser⁵⁶–Tyr⁸¹ are also shown to contribute to neurofascin-ankyrin interactions. Mutation E73Q, which has a minimal alteration of the side chain, greatly impairs ankyrin binding activity, whereas a triple mutation of D57N/D58N/D62N resulted in no loss of ankyrin binding activity. These data provide strong evidence for a high degree of specificity in the interaction between neurofascin and ankyrin.

The new assay for evaluation of neurofascin-ankyrin interactions in co-transfected 293 cells is rapid and effective, although qualitative, and has several potential applications. This assay could be used to screen various point mutations in ankyrin as well as in the L1CAM family of cell adhesion molecules for their effects on ankyrin-L1CAM interactions in living cells. The membrane recruitment assay could also be used to identify potential proteins such as protein-tyrosine kinases and protein-tyrosine phosphatases that are involved in regulation of association of neurofascin with ankyrin (11). The clinical importance of ankyrin-L1CAM interactions has been exemplified by the point mutation Y1229H in the human L1 gene. Moreover, the membrane recruitment assay could be applied to screen pharmaceutical drugs that can modulate ankyrin-L1CAM interactions and perhaps cause developmental defects in the nervous system. Nonetheless, the assay has drawbacks due to lack of quantitative information and does not provide a dissociation constant (K_d) describing the affinity of association of mutant neurofascins with ankyrin. In addition, without a careful control of the amount of cDNAs used in co-transfection experiments, one species of cDNA could be overexpressed and

complicate subsequent analysis. By using the same type of vector, the same promoter and 3'-untranslated region, and carefully controlled cDNA ratios, our results can be successfully reproduced.

Previous studies of ankyrin-neuroglial interactions in *Drosophila* S2 tissue culture cells have demonstrated that ankyrin selectively associates with neuroglial at sites of cell-cell contact but not other regions of the plasma membrane (10, 12). These observations suggest that recruitment of ankyrin by neuroglial requires activation of neuroglial by extracellular interactions. However, our study demonstrates that recruitment of transfected ankyrin to the plasma membrane of cultured human embryonic kidney cells (293 cells) is solely governed by the presence of the cytoplasmic domain of neurofascin localized at the plasma membrane and is independent of whether the co-transfected cell is isolated or has contact with other cells. The basis for these differences may reflect specialized behavior among L1 CAM family members, differences between S2 cells and human 293 cells, and differences between 270-kDa ankyrin_G and the ankyrin expressed in S2 cells.

An unexpected result of this study is identification of the cytoplasmic domain of neurofascin as a dimer in solution. The effects of dimerization of neurofascin on ankyrin binding and on lateral organization of neurofascin in the plane of the membrane have yet to be determined. Since the ankyrin-binding sequence from Ser⁵⁶–Tyr⁸¹ does not contribute to dimerization and sequences outside the Ser⁵⁶–Tyr⁸¹ sequence do not contribute to ankyrin binding, it is likely that formation of neurofascin dimers does not directly affect ankyrin binding, at least at the qualitative level detectable in the membrane recruitment assay. However, dimerization of neurofascin provides a potential mechanism for formation of neurofascin-ankyrin polymers. Considered together with the multiple binding sites for neurofascin in ankyrin_R (13, 14), the existence of neurofascin dimers

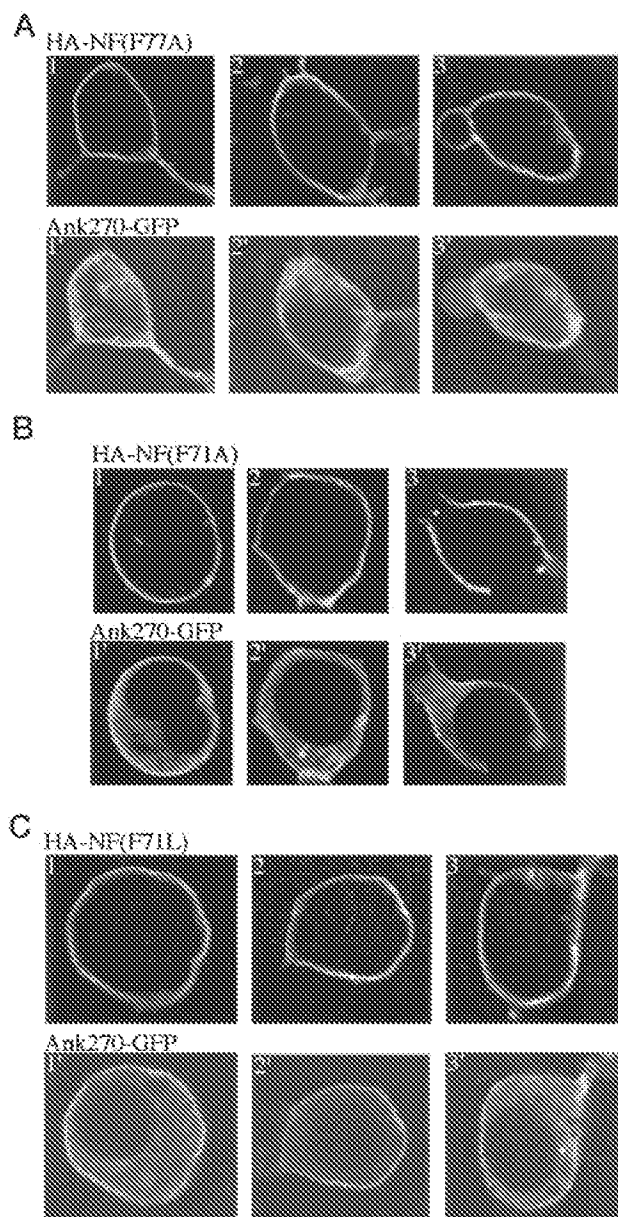


FIG. 10. Other conserved aromatic residues are involved in neurofascin-ankyrin interactions. Mutation F77A (A) and mutation F71A (B) impair membrane association of co-transfected Ank270-GFP. C, mutation of F71L, compared with mutation F71A, does not significantly improve membrane recruitment of co-transfected Ank270-GFP. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 are immunostaining of the HA epitope of mutant neurofascins. 1', 2', and 3' are the GFP fluorescence of co-transfected Ank270-GFP.

implies that neurofascin and ankyrin are able to form lateral complexes containing multiple copies of neurofascin, ankyrin, and possibly other ankyrin-binding membrane proteins. These complexes could be further immobilized by coupling to the spectrin-based membrane skeleton through the spectrin-binding domain of ankyrin. The ability to form such large immobilized complexes between ankyrin and neurofascin could be important for the assembly of specialized membrane domains such as axon initial segments and nodes of Ranvier, where these proteins are localized (16).³

Lack of discernible secondary structure in the sequence Ser⁵⁶–Tyr⁸¹ indicates that effects of various deletions and mu-

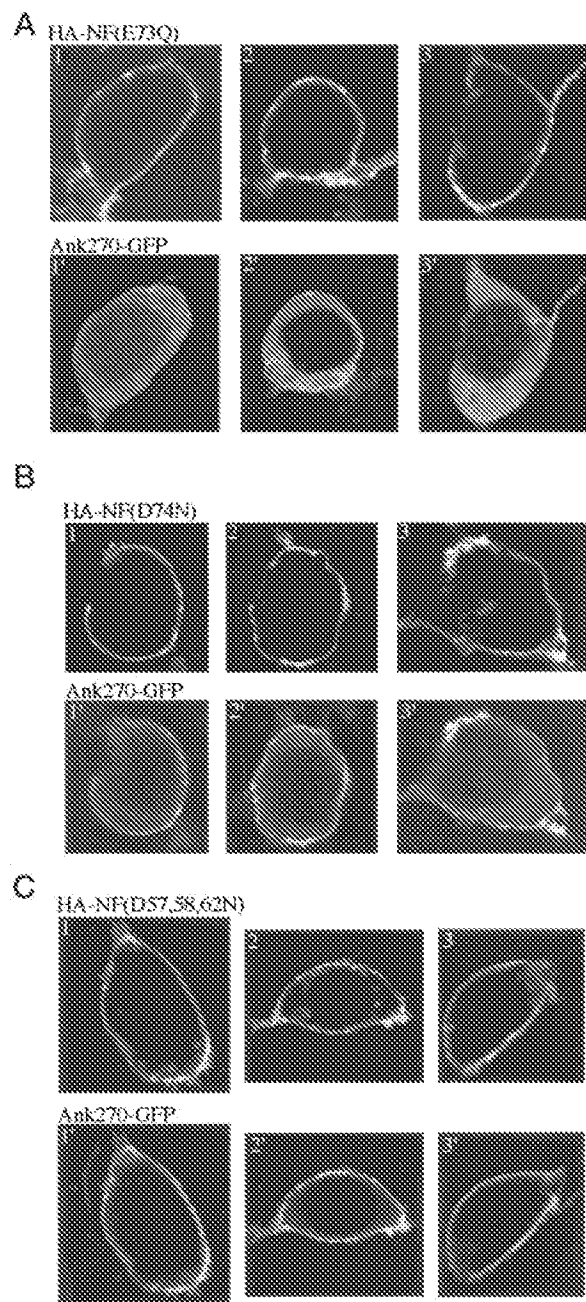


FIG. 11. Conserved negative charges are involved in neurofascin-ankyrin association. A, mutation E73Q greatly diminishes the ankyrin recruiting activity. B, mutation D74N has a moderate deteriorating effect on membrane recruitment of co-transfected Ank270-GFP. C, mutation with Asp⁵⁷, Asp⁵⁸, and Asp⁶² changed to asparagine keeps full ankyrin-recruiting activity. 1 and 1', 2 and 2', and 3 and 3' represent the same transfected cells, respectively. 1, 2, and 3 are immunostaining of the HA epitope of mutant neurofascins. 1', 2', and 3' are the GFP fluorescence of co-transfected Ank270-GFP.

tations in this sequence on ankyrin binding are more likely exerted by direct perturbation of the contact site with ankyrin rather than by disrupting the conformation of the cytoplasmic domain of neurofascin. Atomic structure of ankyrin repeats of the transcription factor GAPB- β revealed that the protein binding site in ankyrin repeats is configured as a tandem array of extended loops, where the tips of these loops provide the binding interface (17). Extrapolation of this information regarding the ankyrin repeats of GAPB- β to ankyrin suggests the sequence Ser⁵⁶–Tyr⁸¹ in the cytoplasmic domain of neurofascin also is in contact with predicted loops. This prediction ulti-

³ Zhang, X., and Bennett, V. (1998) *J. Cell Biol.* **142**, 1571–1581.

mately can be evaluated by determining the structure of ankyrin-neurofascin complexes. It may also be possible to mutate ankyrin at predicted loop sites and "repair" loss of binding of certain neurofascin mutations as well as create ankyrins specifically lacking neurofascin binding activity.

In summary, this study presents a structural and functional analysis of the interaction between ankyrin and the cytoplasmic domain of neurofascin in living cells. Since the ankyrin-binding site is conserved among members of L1CAM family of cell adhesion molecules, our results with neurofascin are likely to apply to other L1CAM molecules.

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